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Can we build synthetic, multicellular systems by controlling developmental signaling in space and time?

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Abstract

Using biological machinery to make new, functional molecules is an exciting area in chemical biology. Complex molecules containing both “natural” and “unnatural” components are made by processes ranging from enzymatic catalysis to the combination of molecular biology with chemical tools. Here, we discuss applying this approach to the next level of biological complexity—building synthetic, functional biotic systems by manipulating biological machinery responsible for development of multicellular organisms. We describe recent advances enabling this approach, including: i) recent developmental biology progress unraveling the pathways and molecules involved in development and pattern formation, ii) emergence of microfluidic tools for delivering stimuli to a developing organism with exceptional control in space and time, iii) the development of molecular and synthetic biology toolsets for redesigning or *de novo* engineering of signaling networks, and iv) biological systems that are especially amendable to this approach.

Introduction

Developmental biology is making tremendous progress in describing the mechanisms that coordinate developmental programs and lead to formation of cells of the correct type at the right place at the right time [1-8]. Concurrently, a revolution in micro- and nanoscale engineering and microfluidics is enabling unprecedented control over the cell's microenvironment [9,10]. It is patently obvious that humans do not make machines the way nature makes them. As both chemical technology to interface with biological systems on the micro-scale (“microchemical interface technology”) and our knowledge of developmental biology become more sophisticated, a fundamental question becomes unavoidable: with the right gene constructs and advanced microchemical interface technology, can multicellular development be utilized as a technology to fabricate machines (functional, synthetic biological systems)? This is a fundamental, open question at the intersection of information science, engineering, chemistry, and biology. For biologists and chemists, such technology would present new ways of interrogating the control systems that transform a single cell into a whole organism. For engineers, this could open the door to a whole new way of making machines, allowing us to adopt the methods by which nature fabricates and assembles biological organisms.

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Much of the effort at the interface between the science of development and the engineering of microchemical interface technology is focused on regenerative medicine [11,12] and, to a lesser extent, microbiology [13-15]. More recently, synthetic biologists have begun to treat the cell, from the 'bottom up', as an entirely *de novo* engineered system [16-18]. These efforts have largely been confined to clonal populations of prokaryotes (ie. plates of single bacterial cells expressing identical engineered gene constructs). However, this review will not focus on the extensive efforts in regenerative medicine; excellent reviews exist [11,12].

Alternatively, we ask a different question: can the biological development of a complete organism be co-opted to make cell-based machines, including those for non-medical uses? If the goal is to make a new biological system (or modify an existing one), then many of the issues faced in tissue engineering and regenerative medicine are irrelevant, including the clinical applicability and translation from animal models to humans. Historical analogies exist: understanding enzyme kinetics fundamentally changed medicine and pharmacology, but that understanding was also foundational to chemical engineering and industrial catalysis. In addition, most organisms are not mammals; there is an abundance of 'simpler' multicellular systems to interface with and to modify, ones that might serve as starting points for fabrication-oriented efforts [19]. This review will cover recent work in multi-cellular signaling, the latest technologies to interface with developing biological systems and end with a set of sample biological systems that might serve as motivation for this nascent area.

Chemical signals guide multicellular development

Every time a tree or a flea or a human reproduces, a complex program is set in motion that orchestrates development in both space and time to fabricate a new organism [1,2]. For more than a century, developmental biologists and chemists have worked to unravel—to reverse engineer—the rules and mechanisms that organisms use to fabricate themselves. In single cells, thousands of genes encode for products along pathways that regulate, consume, produce and transduce; they allow the cell to sense and respond to stimuli with webs of chemical feedback [3,4,20]. These pathways also enable cells to coordinate with each other. By exchanging chemical, mechanical and other information cells can influence the states of the cells near them. Knowledge of these pathways provides a number of cues to begin engineering or re-engineering the course of development of biological systems. While even a cursory review of developmental mechanisms [2,3] is well outside the scope of this work, we illustrate the principal ideas with three common communication mechanisms that can be readily coupled to mirochemical interface technology and can be manipulated beyond traditional genetic perturbations.

The first mechanism relies on gradients of diffusible signals. For example, the Bicoid protein, a classic morphogen, forms a gradient along the anterior-posterior axis of the developing *Drosophila* embryo and is responsible for the formation of head structures. A high concentration of Bicoid at the anterior pole of the embryo leads to expression of the *hunchback* gene. The embryo is subsequently patterned in progressively finer features by gradients of shorter ranges formed by the products of gap genes and pair-rule genes [1]. In the simplest models, chemical gradients are formed by simple diffusion and are interpreted by threshold responses. However, recent work [3,4,21] suggests that passive diffusion may not be sufficient to explain formation of these gradients and that these gradients may be interpreted in ways more complex that a simple threshold response. This work raises the possibility that active transport of morphogens is involved and provides scientific and engineering opportunities for microchemical interface technology.

The second mechanism involves coupling of signaling molecules and convective fluid flow. For example, cilia generate flow that transports developmental signals from cell to cell. This

mechanism is important in the retinoic acid-mediated left-right symmetry breaking in vertebrate development [22] and in signaling gradients that control migration of neurons in the development of the mouse brain [23]. Remarkably, cilia also respond to externally generated flow and polarize. Cilia both generate flow and respond to the flow around them, creating a feedback loop that is essential for coordinating their activity and organizing development [24]. Such convective transport has two clear advantages over transport by simple diffusion: convective flow can rapidly transport signals over long distances, and transport can be directional.

A third mechanism relies involves the response of cell-surface molecules to stimuli presented by other surfaces, such as the extracellular matrix (ECM) or the surfaces of other cells. This mechanism can be explicit, as when a signal from a neighboring cell controls a cell's fate, or it can be implicit, where surface signals provide context for interpretation of soluble signaling molecules. It is increasingly clear that careful manipulation of the surfaces that contact a cell is essential for the control of developmental processes.

These mechanisms are certainly not all-encompassing, as other factors affecting development could be directly manipulated with microscale systems. One example is the response of cells to mechanical cues [25,26], presumably transduced via tension sensed by the cytoskeleton or membrane structures. This mechanism may control proliferation, differentiation, and activity of cells in a number of systems. Additional examples include electrical cues [11], illumination (as in development of fertilized eggs of brown alga *Fucus*), and perhaps even magnetic fields [27]. Nevertheless, these mechanisms provide clues to how developmental pathways could be manipulated by using microchemical interface technology.

Multicellular signals and pathways can be experimentally altered

As developmental biology has progressed from observing to manipulating, genetic manipulation has become a cornerstone of the field. Through genetic manipulation, gene networks that are sufficiently well understood and modeled may be used to control development [28]. Controlling development by physical manipulation also has a distinguished history in developmental biology. For example, the role of cytoplasmic signaling molecules, like the morphogen Bicoid, was confirmed by physical manipulation of a developing *Drosophila* embryo. Mechanical transfer of cytoplasm, and the signaling molecules therein, from the anterior to the posterior of the embryo gave rise to a head structure in place of a tail structure. In addition, microinjection of purified signaling molecules or small interfering RNAs for genes responsible for production of signaling molecules allows rapid testing of developmental hypothesis. Such experiments involving physical manipulation may also enable real-time control of developmental processes and provide access to additional phenotypes. Combination of physical and genetic or chemical manipulation may be especially powerful, as demonstrated by creating light-sensitive channels that can be triggered in the brain [29,30] [31]. Microchemical interface technology, especially in combination with genetic manipulation, may bring these experiments to a new level of spatial and temporal control, providing exciting opportunities for both science and engineering.

Microscale chemical interface technology may enable organism-wide re-direction of developmental programs

Recent breakthroughs in microfluidics and microfabrication are providing unprecedented levels of spatial and temporal control of chemical environments. These breakthroughs are fueled in part by soft lithography—a set of techniques that moved microtechnologies from specialized clean rooms into biological and chemical laboratories. We will not attempt to repeat

the extensive reviews on the subject [9,32], but rather, we emphasize that these technologies may be used to control the developmental mechanisms outlined above.

First, chemical gradients can be created easily by using laminar flow concatenators [33,34], and these gradients may be transferred to gels and surfaces [12,35]. In addition, ‘pixel-style’ devices for discrete, two-dimensional dosing are just emerging for the generation of complex, dynamic gradients. Such devices have already been utilized to dose neurotrophic agents, chemotactic compounds, differentiation signals, and even small signaling molecules such as oxygen [36,37] (Figure 2). Second, microfluidics also allows exquisite control of fluid flow; on-chip microfabricated valves and pumps can start, redirect and stop fluid flow at will [9, 38,39]. Third, surface chemistry can also be chemically controlled to orchestrate developmental processes. Surfaces can be created with small molecules and proteins in controlled densities, orientations and in a controlled background [12,40]. Dynamically switchable surfaces are being rapidly developed as well [41,42]. All of these methods could be used to control development, with high resolution in space and time, by delivering endogeneous ligands and proteins, or by “drugging” developmental pathways by adding small molecules that modulate endogenous players in a manner well-controlled in space and time.

Simpler multicellular systems may provide templates for multicellular fabrication

Provided with sufficiently advanced microchemical (and possibly, mechanical, electrical, thermal, or optical) interface technology, are there existing multicellular systems that can be modified in useful ways? Are existing organisms too complex or lack the plasticity necessary for modification? Among the well-studied developmental biology animal models, including the fruit fly (*Drosophila melanogaster*), the zebrafish (*Danio rerio*), the sea urchin (*Arbacia punctulata*), and the chicken (*Gallus gallus*), some systems are more amenable to chemical manipulation. The zebrafish, for example, is transparent, develops around a simple sphere (the yolk), and develops normally even if the impermeable chorion is removed [43]. However, simpler models may provide even better substrates for building functional biological machines.

The millimeter scale *Hydra vulgaris* and its close relatives are nature's simplest multicellular organisms possessing a neural net [1,44]. A hydra has no central nervous system. Instead, it has a web of neurons that link chemical and mechanical sensors to primitive musculature, a system sophisticated enough to enable opportunistic feeding on tiny animals wandering into its tentacles. Hydra is much simpler than a mammalian system in a number of ways. It has two (not three) dermal layers, where the outer skin cells serve as both epithelia and enervated muscle. The neurons of the hydra can be stimulated locally and globally with simple electrodes. In addition, the hydra can reproduce by budding. If separated into fragments as small as a few cells, most fragments re-organize themselves into appropriate dermal layers, where cells divide, migrate, and correctly re-form a new hydra in several days [45]. Gradients of chemical signals have long been implicated in establishing and maintaining the hydra's body plan, and several recent chemical screening efforts have been aimed at identifying putative signaling compounds and their roles [46]. How far could a hydra's geometry and neuron-musculature be re-patterned by using a microchemical interface device? Are genetic modifications required? Given recent interest in hybrid metal-muscle devices, the hydra presents an attractive alternative to mammalian muscle constructs [47].

Volvox are colonial green algae which assemble into spheroids of tens to thousands of cells. The line between microorganism colony and multicellular organism blurs as one examines the spectrum of *Volvox* sub-species. In the larger organisms, cells arrange themselves precisely within an extracellular matrix, differentiate into somatic and reproductive cells, collectively locomote towards light, reproduce new spheroids in a coordinated fashion, and are capable of

sexual reproduction with other colonies [48]. Moreover, the sex-inducing pheromone of *Volvox carceri* is one of the most potent signaling compounds known; a 100 aM concentration is sufficient to engage the sexual reproduction pathway [48]. Could *Volvox* be a template for chemically-modulated self-assembly? A recent result suggests that extracellular, matrix-mediated self-assembly can be used to form simple multicellular aggregates similar to those seen in Volvox [49].

A more immediately useful system may be present in vascular plants. It has long been known that plant vasculature is assembled through a combination of chemical signaling and apoptosis, programmed cell death [50]. The prevailing hypothesis is that the tips of growing plants emit auxin which is transported by downstream cells towards the roots. Cells experiencing the highest auxin concentrations reinforce their walls (with lignin and other compounds), form connections to nearby cells undergoing the same process, and finally commit suicide, leaving networks of empty vessels through which water and nutrients flow. This process remains active into adulthood; if the vasculature is wounded, auxin builds up locally and nearby cells are recruited to form new vascular channels [51]. Exogenously applied, auxin is known to trigger vascular growth towards the source [52]. In this fashion, plants have solved three long-standing engineering problems that still plague modern microfluidic systems: fluidic interconnections across scales ranging from the micro- to the macro- scale (plant vasculature links the smallest leaf capillaries to the largest trunk arteries), the ability to withstand large pressures without generating bubbles through embolism, and high velocity fluid transport without active pumps.

Additionally, a plant's chemical processing and metabolism is mediated via the vasculature. Lastly, it is a plant's vasculature in dead form, the secondary xylem, that gives wood its amazing structural range from balsa's lightness to bamboo's hardness [53]. Could we co-opt this system to microfabricate vascular networks?

It may be that existing multicellular systems are too complex or too developmentally inflexible for microchemical control of their developmental machinery. For example, microfluidic interface technology has previously been used to show that the development of the *Drosophila* embryo is robust under the environmental perturbation of a temperature step (Figure 3). When the two halves of the embryo are maintained at different temperatures, the two halves develop at different rates [54,55]. Nevertheless, when the temperature step is removed sufficiently early the embryo resynchronizes the two halves and proceeds to develop normally. Future experiments utilizing microchemical interface technology may enable understanding of the mechanisms responsible for robustness of development and may uncover the limits beyond which developmental programs cannot be perturbed. If so, the answer may lie in the approaches of synthetic biology. Could we take simple microorganisms, add the right chemical signaling genes, and direct their growth with microchemical interface technology [13]? A recent result demonstrates that prokaryotes can be genetically modified to produce synthetic pattern formation [17]. A number of robust pattern generation systems have been studied for decades, both at the experimental and theoretical level. These include Turing reaction-diffusion systems [56-58], simple gradient generators [1,59], and chemotaxis models. Could synthetic, addressable pattern generators be inserted into prokaryotes? This is a completely open question.

As with all interventions of organismal development, ethical questions arise. While an adequate ethical discussion is beyond the scope of this review, most, if not all, concerns are already part of the healthy debates arising from both synthetic biology and regenerative medicine efforts [60,61].

Conclusions

Advances in microchemical interface technology, chemical tools, synthetic biology, and developmental biology are provoking a fundamental question: to what extent can multicellular development be used as a technology to make machines? It is too early to tell whether fabrication methods based on such an approach would yield useful devices or if they lie entirely in Dr. Alphonse Mephisto's domain. We are encouraged by the successes of using biological machinery to make new natural and unnatural molecules, and by coupling between microfluidics and chemistry to construct functional reaction networks [62-64]. Regardless of the success of such engineering endeavors, sophisticated microchemical interface technology are interesting in their own right. Such tools will give developmental biologists new ways of understanding the mechanisms that robustly transform a cell into an organism. If multicellular development is amenable to significant re-design and control, this could open the door to an exciting new way of making machines, allowing us to adopt the methods by which nature fabricates and assembles biological organisms.

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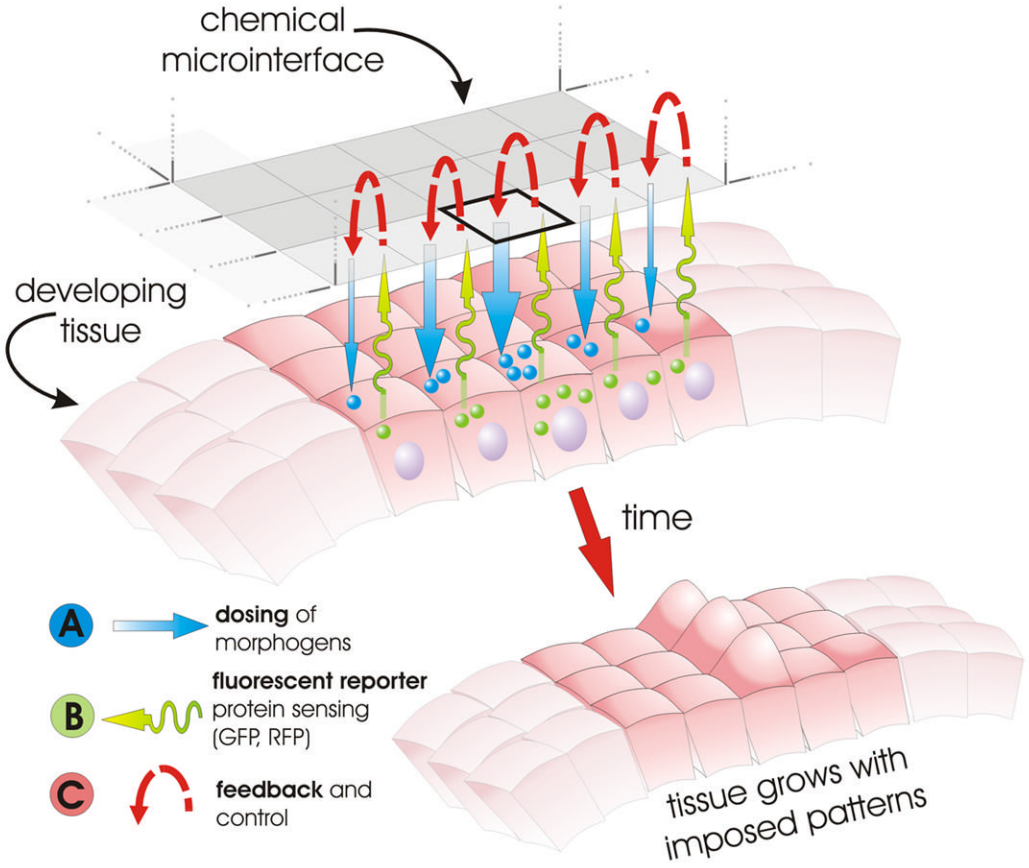

Figure 1.
A cartoon illustrating chemical microinterface for controlling, in real time, concentration of morphogens across developing tissue. Morphogens are delivered with high spatial and temporal resolution (blue arrows). Their effect is read out using integrated fluorescent reporters (green arrows) and dozing of morphogens is adjusted using feedback control mechanisms to achieve the desired differentiation and growth of tissue.

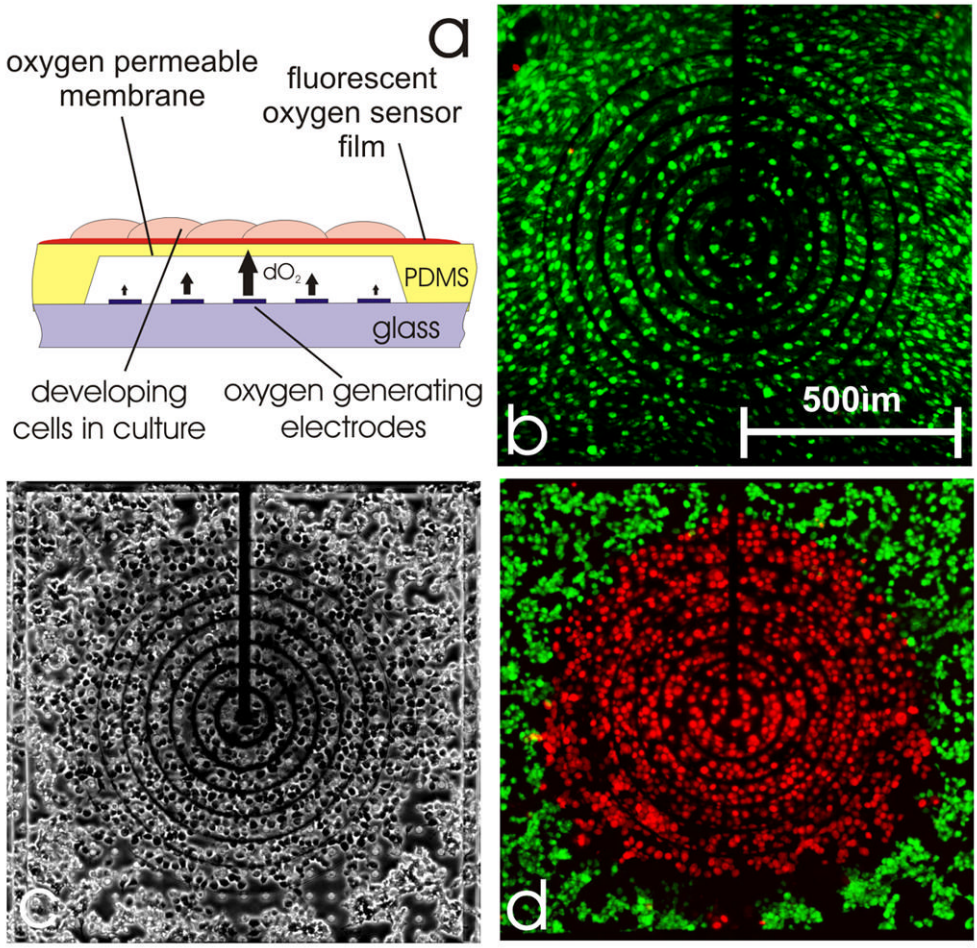

Figure 2.

A chemical microinterface for controlling oxygen gradients in developing cells; from reference [37]. **a)** Cells are cultured over an oxygen permeable membrane. Oxygen-generating electrodes are independently controlled so as to deliver more or less oxygen to different parts of the culture. **b – d)** Hyperoxia induced apoptosis in C2C12 myoblasts. **b)** LIVE/DEAD ® image of myoblasts after 72 hours in anaerobic chamber (95% N_2 / 5% CO_2) with continuous normoxic oxygen delivery from microinterface. **c)** White light microscopy 2 hours after applying a localized, circular hyperoxic (~40%) pattern of oxygen. **e)** Fluorescent image of c) with LIVE/DEAD ® stain.

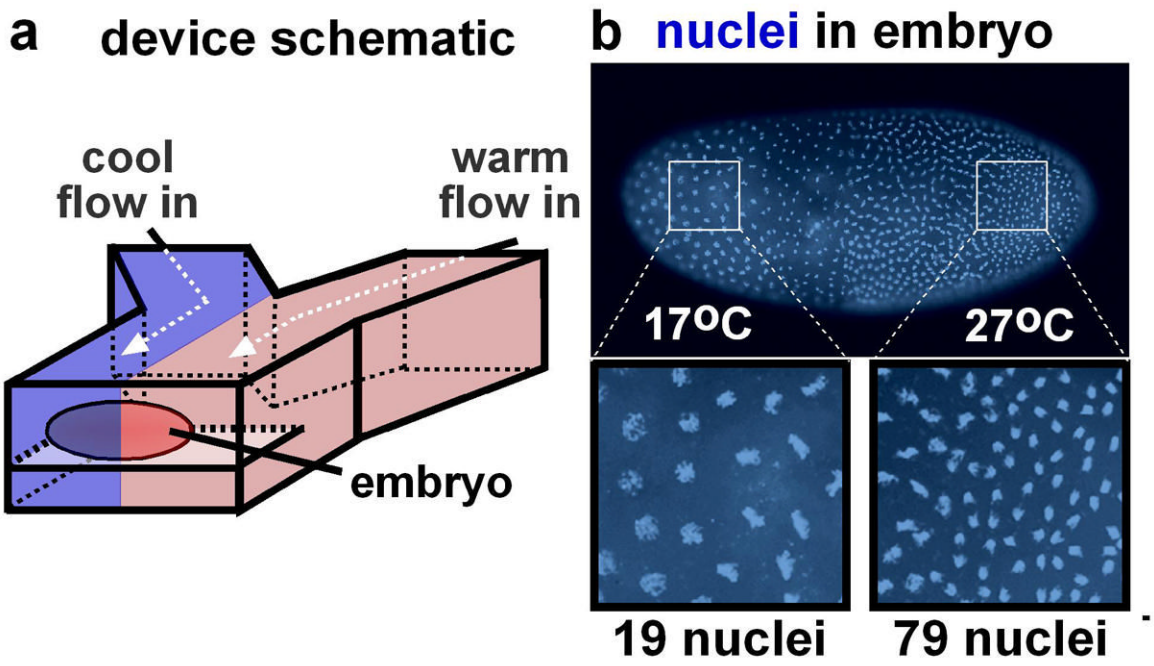

Figure 3.
Microfluidic interface technology to control the development of a *Drosophila* embryo in space and time (adapted from reference [54]). a) Schematic drawing of a temperature step around a live embryo in a microfluidic device. b) As visualized by the difference in nuclear density in the two halves of the embryo, the difference in temperature affects the rate of development in each half of the embryo, with the cool half developing more slowly that the warm half.